Pergamon

Total sterols in root-knot nematode *Meloidogyne incognita* infected cotton *Gossypium hirsutum* (L.) plant roots*

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Two near-isogenic breeding lines of *Gossypium hirsutum* (L.) (upland cotton) were inoculated with eggs of *Meloidogyne incognita* (Kofoid and White) Chitwood (RKN) in greenhouse pots. One line was susceptible (ST-213) and the other was resistant (81-249) to RKN. Eight days after inoculation, roots of plants and their controls were analyzed for total sterols by GLC and GLC–MS. The total content in roots of uninoculated roots of susceptible plants was slightly higher than in the roots of uninoculated resistant plants, but significantly lower after inoculation. By comparison, the sterols in roots of the resistant line were significantly higher at 8 days after inoculation. The major sterols in both susceptible and resistant lines were stigmasterol, sitosterol and campesterol, with a lesser amount of cholesterol and a trace of stigmastanol. Because the amounts and distribution of the sterols were similar in susceptible and resistant lines, they do not appear to be a factor associated with RKN growth and development. Sitosterol was the most abundant sterol in RKN eggs which also contained lesser amounts of cholesterol and stigmasterol. However, no stanols were found in RKN eggs.

Introduction

The root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood (RKN) is a sedentary endoparasite that retards growth and development of cotton *Gossypium hirsutum* (L.) by attacking the root system, causing galling,

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stunting, and other adverse effects (Shepherd, 1974; Shepherd *et al.*, 1989). Several breeding lines of cotton with very high levels of resistance have been selected (Shepherd, 1974; Shepherd *et al.*, 1989). Creech *et al.* (unpublished data) have shown that although RKN initially infects these resistant lines, development beyond a swollen secondary juvenile stage ceases by 8–10 days following inoculation.

Veech (1978) found increases of methoxylated terpenoid aldehydes in roots of *G. barbadense* cotton following infection with RKN. Hedin *et al.* (1984) reported that the terpenoid aldehyde content of *G. hirsutum* cotton roots of a RKN resistant line was higher initially and increased faster after inoculation than that of a susceptible line. However, later work (Khoshkhoo *et al.*, 1994), indicated that roots of

some resistant lines have very low intrinsic terpenoid aldehyde contents. Thus, increases in terpenoid aldehydes are not necessarily associated with resistance to RKN. Consequently, a search for other factors of resistance appeared appropriate.

All nematodes that have been cultured on chemically defined media have been found to require a sterol source as an essential nutrient. Plant parasitic nematodes (including the RKN) have additional interesting metabolic characteristics including the ability to produce stanols and to dealkylate C-24 sterols. Unlike certain free-living nematodes, they do not synthesize significant quantities of 7-dehydrocholesterol from the prominent phytosterols, sitosterol, campesterol, and stigmasterol (Chitwood *et al.*, 1987a,b).

The earliest report of sterols in cotton buds was by Thompson *et al.* (1970), who found 60% of the total sterols in the free state, with the remainder present as esters or glycosides. The major sterol found was sitosterol, with lesser amounts of campesterol, stigmasterol, and cholesterol. The sterols and their derivatives constituted 0.019% of the fresh weight of buds. Svoboda and Rebois (1977) reported a fairly similar distribution of sterols in roots of the cotton cultivar Auburn 56; cholesterol 0.6%, 24-methylcholesterol (campesterol) 5.1%, 24-ethylcholesta-5,22E-dienol (stigmasterol) 43.4%, and 24-ethylcholesterol (sitosterol) 48.5%.

Chitwood *et al.* (1987c) and Chitwood (1991) identified as many as 23 sterols in RKN eggs harvested from eggplant *Solanum melongena* (Black Beauty) and chili pepper *Capsicum frutescens* (Anaheim). The major sterols reported were cholesterol (5.6%), cholestanol (2.6%), 24-methylcholesterol (8.6%), 24-methylcholestanol (7.7%), stigmasterol (4.1%), sitosterol (38.7), and 24-ethylcholestanol (23.8%). Evidently, there has been no report of sterols from RKN eggs harvested from cotton roots.

Because nematodes require a sterol source as an essential nutrient, this study was conducted to determine whether the lack of RKN development in our resistant lines was attributable to the absence of nutritionally required phytosterols otherwise present in the susceptible lines. We analyzed total root sterols from control and RKN inoculated plants of both resistant and susceptible lines, choosing a post-inoculation time (eight days) where differences in development of the nematode just begin to diverge in the two lines (Creech *et al.*, unpublished data). For comparison, the sterols of RKN eggs were also analyzed.

Materials and Methods

Inoculation and harvesting of cotton roots for analysis

Egg inoculum was obtained from previously RKN (Race 3) infected susceptible 'S' cotton by the procedures of Shepherd (1979), who had earlier isolated and characterized the RKN (Shepherd, 1974; Shepherd *et al.*, 1989). Plants were inoculated with RKN eggs as follows: plastic pots (7.0 × 7.6 cm) were placed on greenhouse benches and filled with methyl bromide-fumigated soil (Wickham sandy loam soil, a fine loamy, mixed thermic Typic Hapludult). Approximately 10,000 eggs were deposited in a 3-cm-deep centered hole in the soil of each pot, wetted, and 3-day seedlings were then planted in each pot. Near isogenic susceptible and resistant cotton lines were compared. The susceptible line hereafter identified as 'S' was the ST-213 cultivar. The resistant line, 81-249, hereafter identified as 'R' (Creech, unpublished data), was an experimental line backcrossed twice to the ST-213 parent. The mean number of *M. incognita* eggs per plant produced on 40-day-old cotton plants was 101,750 for the susceptible ST-213 line and 1133 for the resistant isoline 81-249. The plants were grown in a greenhouse at 28°C. Eight days after inoculation of the plants at which time differences in development between the 'S' and 'R' lines due to the RKN were beginning to occur, the roots were harvested and cleaned thoroughly with a low pressure water spray to remove adhering soil, freeze-dried, ground through a 40-mesh screen, and stored at −70°C until analyzed. Three replicates were analyzed.

Sterol analyses were also carried out on RKN eggs. They were harvested from approximately 20-day-old egg masses on roots of plants that had been inoculated 40 days earlier using the procedures of Shepherd (1979) as recently modified by Tang *et al.* (1994). The eggs were collected 2–3 days before hatching and were predominantly at the J-1 stage. The eggs were washed in distilled water (1000 g for 5 min, 2–4 ×) and resuspended prior to being freeze-dried. Total numbers of nematodes were estimated by counting aqueous aliquots with a microscope. Based on the weights of the nematodes after freeze-drying, the average dry weight per egg was calculated as 32 ng. A second approach to estimation of weight was obtained by microscopic measurement in which the eggs were considered to be cylinders of $L = 0.00836$ cm and $r = 0.00365$ cm (84 μm long and 73 μm in diameter). Using $V = L \times \pi r^2$, and assuming the same density as water, the average wet weight per egg was calculated as 86.8 ng and the dry weight was 17.4 ng, of the same order

of magnitude as that based on the method that employed freeze drying. These analyses were performed in duplicate.

Sterol analysis

Total sterols were extracted, saponified, and purified by procedures modified from those of Chitwood et al. (1987b). A 2.2 g sample of dried root tissue was extracted three times with 40 ml of chloroform/methanol: 2/1, V/V, and the extract was partitioned against 0.85% aq. NaCl (20% by volume of organic phase). The lower layer was saved, evaporated to near dryness, taken up to 4% methanolic KOH, and refluxed 4 hr. After addition of 25 ml H_2O, the reflux mixture was extracted with 25 ml portions of hexane four times, washed with H_2O, and dried over anhydrous Na_2SO_4. The hexane layer was evaporated to 1 ml and chromatographed on a 1 × 18 cm Baker Silica Gel (60–200 mesh, 6 g) column in which the silica had been packed as a slurry with hexane. The lipids were eluted with 50 ml volumes each of 0, 2, 5, 10, 22, 30, 50, and 100% ethyl ether in hexane. The free sterols were found in the eluates obtained by chromatography with 22, 30, and 50% ethyl ether. These procedures were similar to those of Chitwood et al. (1987b), who used Florisil (J. T. Baker, Phillipsburg, NJ), and TLC of eluates confirmed the general similarity of elution patterns. Stanols were sought but not found in the 10% or adjacent ethyl ether fraction. For quantification, aliquots of the three fractions (22, 30, and 50% ethyl ether) from each original sample were analyzed by GLC-MS, related to the relative responses of internal standards, summed, and finally expressed as mg/g of sample.

Sources of sterol standards

The following sterols were used to calibrate a 30 m × 0.32 mm i.d. (0.25 μm layer thickness) J and W DB-1 column: (J and W Scientific Inc., Rancho Cordova, CA) cholesterol, cholestanol, desmosterol, lathosterol, campesterol, stigmasterol, sitosterol, stigmastanol (sitostanol), and cycloartenol. They were used without further purification. Additional quantities of some of these sterols were obtained from Sigma Chemical Co., St. Louis, MO. Base line separations for each were achieved and relative retention times (RRT's) were calculated.

Instrumentation

GLC-MS was performed with a Hewlett-Packard 5989 GCMS system interfaced to the previously described GLC column which was programmed from 200°C at 15 deg/min with a 1 min hold to 280°C, then 2 deg/min to 300°C with a 5 min hold at 300°C. The carrier was

helium at 15 psi. Splitless injections of 0.25 μl were made with a Hamilton 1 μl syringe. Injector temperature was 250°C, interface temperature was 300°C, source temperature 250°C, analyzer 100°C. Electron impact spectra were acquired at 70 eV from 33 to 550 AMU in 1.2 sec. GLC-MS quantification was by area count from the total ion chromatogram.

GLC-FID (flame ionization detection) was performed on a Hewlett-Packard series 2 GC using a HP-3394 integrator. GC conditions were the same except the carrier gas pressure was increased to 20 psi.

Library searches were performed on the 54K NIST library supplied with the Hewlett-Packard GLC-MS. Additional searches were performed using a standalone NIST library (NIST/ EPA/MSDC Mass Spectral Database, Version 3.0). Identifications were based on library matches and comparison to authentic reference material spectra and GC retention time data when available.

Statistical analysis

An analysis of variance was conducted separately for each compound assuming a completely randomized design with a factorial arrangement of TRT (treatment) (2 cotton × 2 TRT). F-Tests for the cotton by TRT interaction are indicated in Table 1. Mean comparisons were made on TRT for each cotton using the LSD at P ⩽ 0.05.

Results

Sterols found in cotton roots and RKN eggs (after saponification) are listed in Table 1. The total sterol content in uninoculated roots of 'S' (ST-213) plants was significantly higher (17%) than in roots of the 'R' (81-249) uninoculated isoline (1.62 vs 1.35 mg/g). The total sterol content in roots of inoculated 'S' plants was significantly lower (34%) than in the roots of uninoculated 'S' plants (1.07 vs 1.62 mg/g). By comparison, the total sterol content in roots of the inoculated 'R' isoline was significantly higher (1.69 vs 1.35 mg/g) at 8 days after inoculation.

Among individual sterols in roots, stigmasterol was the most abundant, followed by sitosterol and campesterol. Only 1–3% of the total sterol content was comprised of cholesterol or isofucosterol along with less than 1% each of stigmastanol, lanost-8-en-3-ol, and desmosterol. In addition, two ketones, stigmast-4,22-dien-3-one and stigmast-4-en-3-one, were found in very low concentrations, but they may be degradation products. GLC and GLC–MS provided additional FID maxima of low abundance that could not be identified.

Stigmasterol, which is the most abundant

Table 1. Total sterols in RKN infected cotton plant roots and RKN eggs (mg/g and percent)

No.	Compound	MW	RRT*	ST-213 Non-Inoc		ST-213 Inoc		81-249 Non-Inoc		81-249 Inoc		Nematode eggs		Cotton × TRT F-value†
				mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	
1.	Cholesterol	386	1.00	0.030A	1.8	0.019C	1.8	0.018C	1.3	0.023B	1.4	0.096	24.0	28.6
2.	Desmosterol	384	1.03	—	—	—	—	—	—	—	—	tr	—	—
3.	24-Methylene cholesterol	398	1.06	—	—	—	—	—	—	—	—	tr	—	—
4.	Campesterol	400	1.11	0.144A	8.9	0.126A	11.8	0.119A	8.9	0.230A	13.6	0.020	5.0	2.4
5.	Stigmasterol	412	1.15	0.861A	53.1	0.466B	43.8	0.755A	56.0	0.798C	47.3	0.076	18.8	28.4
6.	Lanost-8-en-3-ol	428	1.17	0.011A	0.7	0.008A	0.8	0.008A	0.6	0.009A	0.5	—	—	2.5
7.	Sitosterol	414	1.21	0.529A	32.6	0.392B	36.8	0.409B	30.4	0.570A	33.8	0.155	38.6	17.9
8.	Isofucosterol	412	1.23	0.020C	1.2	0.032A	3.0	0.021C	1.6	0.026B	1.6	0.055	13.6	11.6
9.	Stigmastanol	416	1.23	tr	—	tr	—	tr	—	tr	—	—	—	—
10.	Stigmasta-4,22-dien-3-one	410	1.29	0.013A	0.8	0.013A	1.2	0.011A	0.8	0.015A	0.9	—	—	0.2
11.	Stigmasta-4-en-3-one	412	1.37	0.014A	0.9	0.010A	1.0	0.007B	0.5	0.016A	1.0	—	—	3.9
	Total	—	—	1.62A	—	1.066C	—	1.348B	—	1.687A	—	0.402	—	—

*Also trace components at RRT (relative retention times) 1.05, 1.18, 1.25, 1.26, 1.33, and 1.40 in roots.
†Means in same row not followed by a common letter differ significantly based on LSD $P \leq 0.05$.

root sterol, was decreased in the susceptible ST-213 line from 0.86 mg/g in uninoculated roots to 0.47 mg/g in inoculated 'S' roots. The stigmasterol concentration in the inoculated and uninoculated 'R' isoline were similar (0.80 vs 0.75 mg/g). The two other relatively abundant 24-alkyl sterols, sitosterol and campesterol, were present at lower concentration in inoculated 'S' roots by 26 and 13%, respectively.

The total sterol content of nematode eggs was 0.40 mg/g of dry weight (Table 1). The most abundant sterol in RKN eggs (Table 1) was sitosterol (38% of total) followed by cholesterol (24%), stigmasterol (19%), isofucosterol (14%) and campesterol (5%).

Discussion

The total sterol content in uninoculated roots (0.16% on a dry weight basis) was similar to that reported in cotton buds by Thompson et al. (1970) (0.019% of fresh weight). Their reported distribution in buds was about 50% sitosterol followed by campesterol, stigmasterol, and a small amount of cholesterol. Svoboda and Rebois (1977) had reported mostly sigmasterol and sitosterol (43.4 and 48.5%) in uninfected cotton roots. Also present were 0.6% cholesterol, 5.1% campesterol, 2.6% unidentified, and a trace of stanols. Similarly, we found stanols to comprise less than 1% of the total sterols in roots.

Zinoveva et al. (1989) reported that RKN increased total root sterols of two susceptible tomato lines by 39 and 247% after infection, while root sterols decreased by 37 and 39% in two resistant lines after infection. Stigmasterol, the dominant sterol in the roots, underwent the greatest changes, being much higher in the susceptible and decreased in the resistant tomato lines. Thus, the patterns of change we observed in total root sterols in RKN infected cotton roots were opposite to that reported in tomato.

We found the total sterol content of nematode eggs to be 25% of that in cotton roots and somewhat lower than the 0.10–0.16% of dry weight measured in three free-living nematodes (1987b), but higher than the 0.01 mg/g of dry weight in M. incognita and M. arenaria eggs harvested from eggplant and chili pepper (1987b). The sterol composition we measured within eggs differed in M. incognita Race 3 considerably from that reported by Chitwood et al. (1987c) for RKN eggs isolated from eggplant in which sitosterol and 24-ethylcholestanol were the dominant sterols and cholesterol was only 5.6% of the total. Chitwood et al. (1987c) also found significant quan-

tities of cholestanol, 24-methylcholestanol, and 24-ethylcholestanol in eggs.

We were concerned that we were unable to find any stanols in eggs and only traces in cotton roots. However, our GLC system successfully separated two commercially available sterol pairs, cholesterol and cholestanol, and 24-ethylcholesterol and 24-ethylcholestanol. Cholesterol eluted from our GLC column approximately 0.11 min earlier than cholestanol with near baseline separation. Scans obtained at their respective maxima gave no evidence of M/e 388 in the cholesterol peak and no M/e 386 in the cholestanol peak.

We also considered the possibility that in our column chromatographic clean-up regimen, the stanols may have eluted earlier than the sterols. However, no stanols were evident in our GLC–MS analyses. We also considered that our TLC monitoring system may not have visualized the stanols. However, cholestanol and 24-ethylcholestanol also reacted quickly with the anisaldehyde. It is also possible that if the eggs had been harvested from the roots earlier or later than at the stated 40 days after inoculation, stanols would have been detected. However, we used the 40-day time of harvest to evaluate cotton varieties for RKN resistance because it coincides with the achievement of a population maxima (Shepherd, 1974, 1979; Shepherd et al., 1989).

The major objective of this work was to determine if resistance to RKN was associated with a unique root sterol composition, with the idea that resistant plants may fail to provide a suitable source of sterols for the RKN. Although sterols were 17% lower in uninoculated resistant roots, the difference does not seem sufficient to be nutritionally significant. By 8 days after inoculation with RKN, the sterol content of 'S' roots had declined by 34% while that of 'R' roots increased by 17%, even though the relative number of J2 juveniles in the 'R' and 'S' lines are known to be similar at this time (Creech et al., unpublished data). Thus, RKN living in 'R' roots did not appear able to metabolize phytosterols during this period. RKN in 'S' roots may have been able to metabolize the sterols because they were decreased by 34% after 8 days. On the other hand, sterols could also be changed as a result of plant response to general stress, wounding, or changes in host plant sterol biosynthesis as a result of RKN feeding. Also, while it is conceivable that the bound sterols may have been different in 'S' and 'R' plants, the similar distribution of free sterols found after saponification does not seem to encourage the speculation that some sterol, either present or absent, was required or toxic to RKN. Control of RKN growth and develop-

ment in the 'R' line is evidently exerted on some alternative basis.

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